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Interleukin-1α signaling initiates the inflammatory response to virulent *Legionella pneumophila in vivo*

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Abstract

Legionella pneumophila is an intracellular bacterial pathogen that is the cause of a severe pneumonia in humans called Legionnaires' Disease. A key feature of *L. pneumophila* pathogenesis is the rapid influx of neutrophils into the lungs, which occurs in response to signaling via the interleukin-1 receptor (IL-1R). Two distinct cytokines, IL-1α and IL-1β, can stimulate the IL-1R. IL-1β is produced upon activation of cytosolic sensors called inflammasomes that detect *L. pneumophila in vitro* and *in vivo*. Surprisingly, we find no essential role for IL-1β in neutrophil recruitment to the lungs in response to *L. pneumophila*. Instead, we show that interleukin-1α is a critical initiator of neutrophil recruitment to the lungs of *L. pneumophila*-infected mice. We find that neutrophil recruitment in response to virulent *L. pneumophila* requires the production of IL-1α specifically by hematopoietic cells. In contrast to IL-1β, the innate signaling pathways that lead to the production of IL-1α in response to *L. pneumophila* remain poorly defined. In particular, although we confirm a role for inflammasomes for initiation of IL-1β signaling *in vivo*, we find no essential role for inflammasomes in production of IL-1α. Instead, we propose that a novel host pathway, perhaps involving inhibition of host protein synthesis, is responsible for IL-1α production in response to virulent *L. pneumophila*. Our results establish IL-1α as a critical initiator of the inflammatory response to *L. pneumophila in vivo*, and point to an important role for IL-1α in providing an alternative to inflammasome-mediated immune responses *in vivo*.

Introduction

Legionella pneumophila is a gram-negative intracellular bacterial pathogen that is the causative agent of a severe pneumonia called Legionnaires' disease. After inhalation of aerosolized bacteria, *L. pneumophila* can infect and replicate within lung alveolar macrophages. Intracellular replication of *L. pneumophila* in macrophages *in vitro*, and virulence of *L. pneumophila* in animal models, requires a Type IV secretion system (T4SS) called the Dot/Icm system, which secretes bacterial effector proteins into the host cytosol. These effectors, greater than 270 of which have been identified (reviewed in 1), are believed to be critical for establishment of the *Legionella*-containing vacuole, the specialized membrane-bound intracellular compartment in which *L. pneumophila* replicates. In addition to its essential role in facilitating intracellular bacterial replication, the *L. pneumophila* T4SS

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is also associated with the induction of several potent innate immune responses (reviewed in 2).

Legionnaires' Disease is characterized by robust infiltration of neutrophils and other immune cells into the lungs (3–5). Mice depleted of neutrophils exhibit an increased burden of *L. pneumophila* in the lungs (6–9). Furthermore, *in vivo* blockade of the CXCR2 chemokine receptor reduces the number of neutrophils recruited to the lungs of *L. pneumophila* infected mice and increases the lethality of *L. pneumophila* infection (8). Despite the clear protective role of neutrophils in *L. pneumophila* infections, it is also believed that excessive neutrophil influx may be responsible for much of the pathology associated with Legionnaires' Disease (3, 5). Thus, infected hosts require mechanisms to carefully regulate the influx of neutrophils into tissues such that sufficient neutrophils are recruited to mediate pathogen clearance without causing excessive immune pathology. Despite the central role of neutrophils in Legionnaires' Disease, the mechanisms controlling neutrophil recruitment to the lung in response to *L. pneumophila* remain poorly understood.

Previous work has established that neutrophil recruitment to the lung in response to *L. pneumophila* requires bacterial expression of the Dot/Icm T4SS (6). In addition, the interleukin-1 receptor type I (IL-1R), and its downstream signaling adaptor protein, MyD88, are also required (6, 10–13). Toll-like receptors (TLRs), which also utilize the MyD88 signaling adaptor, appear to only have a modest role in neutrophil recruitment to the lung (10–12, 14), suggesting that IL-1R signaling is the main pathway leading to neutrophil recruitment to the lung *in vivo*. Two related cytokines, interleukin-1α (IL-1α) and interleukin-1β (IL-1β), can both signal through the IL-1R. A previous study suggested that IL-1β is critical for neutrophil recruitment in response to *L. pneumophila* (6). It was proposed that infected macrophages generate IL-1β that signals through the IL-1R expressed by airway epithelial cells (AECs). IL-1R signaling in AECs amplifies the initial IL-1β signal by triggering the production of chemokines, such as CXCL1 and CXCL2, which stimulate the rapid and robust recruitment of neutrophils to the lung (6). However, no study has specifically addressed a possible role for IL-1α in mediating IL-1R-dependent neutrophil recruitment *in vivo*, and consequently, the relative role of IL-1α and IL-1β in responses to *L. pneumophila* remains uncertain.

Both IL-1α and IL-1β lack classical signal peptides to target the proteins to the conventional secretory pathway, and the mechanism of their release from cells remains poorly understood. Production of IL-1β appears to require two steps. First, activation of the NF-κB transcription factor results in transcription of *Il1b* mRNA, which is then translated into pro-IL-1β protein. Release of mature IL-1β has then been shown, in most instances, to require the Caspase-1 protease, which cleaves and activates IL-1β into its biologically active form (15, 16). Caspase-1 is itself activated within multiprotein complexes called 'inflammasomes' (17, 18). *L. pneumophila* has been shown to stimulate IL-1β release primarily via the NAIP5/NLRC4 inflammasome that senses bacterial flagellin that is translocated into the host cell cytosol via the Dot/Icm T4SS (19–23).

In contrast to IL-1β, IL-1α does not require proteolytic processing by Caspase-1 in order to be biologically active (24, 25). Nevertheless, in certain instances, inflammasome activation can promote the extracellular release of IL-1α, perhaps as a result of inflammasome-induced cell death (24, 26, 27). However, it is still unclear if the inflammasome is required for IL-1α production in response to bacterial infections *in vivo*. Similar to *Il1b*, the *Il1a* gene can be transcriptionally induced by infection, but IL-1α may also be constitutively expressed in certain cell types (24, 28). Virulent ($T4SS^+$) *L. pneumophila* has been shown to induce IL-1α production by macrophages *in vitro* as well as in lung infections *in vivo* (29). In contrast, Δ*dotA L. pneumophila* mutants, which lack an active T4SS, do not induce IL-1α

in vitro or *in vivo* (29). Nevertheless, the precise mechanism of IL-1α production in response to *L. pneumophila* remains unclear. Previous studies have suggested that T4SS-dependent activation of p38 and JNK MAP kinases are required to induce *Il1a* transcription (29, 30). Activation of MAP kinases by *L. pneumophila* appears to be partially due to a T4SS-dependent inhibition of host protein synthesis (30). Five *L. pneumophila* T4SS-translocated effectors have been identified that inhibit host protein synthesis (31), and *Myd88/Nod1/Nod2*$^{-/-}$ macrophages infected with a strain lacking these five effectors (Δ*5*) exhibit diminished MAP kinase activation and reduced *Il1a* mRNA levels as compared to wild-type-infected macrophages (30). However, infection of WT macrophages with the Δ5 mutant still induces normal MAP kinase activation (30), implying that MyD88/Nod signaling can also contribute. The mechanism by which protein synthesis inhibition results in MAP kinase activation remains unknown, and moreover, it is not clear whether macrophages infected with the Δ*5 L. pneumophila* strain exhibit a defect in release of IL-1α protein.

Here we show that in response to infection with virulent *L. pneumophila in vivo*, IL-1α produced by hematopoietic cells is the dominant cytokine leading to neutrophil recruitment to the lung at early timepoints (0 to 12 hours) after infection. We find that IL-1α and IL-1β act redundantly at later timepoints as neither *Il1a*$^{-/-}$ nor *Il1b*$^{-/-}$ mice have defects in neutrophil recruitment or bacterial clearance in the lung 24 hours post-infection. Interestingly, IL-1α is produced normally in mice lacking both Caspase-1 and Caspase-11, strongly implying that inflammasomes are not required for IL-1α production. Mice deficient in both *Casp1/11* and *Il1a* phenocopied *Il1r*$^{-/-}$ mice, confirming that inflammasomes can compensate for a lack of IL-1α at late timepoints. Interestingly, we did not detect a defect in IL-1α production in macrophages infected with the *L. pneumophila* mutant lacking 5 bacterial effectors that block host translation (Δ*5*). While the Δ5 mutant had no defect in IL-1α production, we find that translation inhibition in concert with TLR activation is sufficient to induce IL-1α *in vitro* and *in vivo*. Taken together with previous studies (29–31), these results suggest that an uncharacterized pathway is responsible for IL-1α production in response to *L. pneumophila* infection *in vivo*. Our results point to a critical role for IL-1α in initiating IL-1R-dependent neutrophil recruitment and inflammatory responses *in vivo* that is complementary to the established inflammasome/IL-1β signaling axis.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee at the University of California, Berkeley.

Mice

Except for bone marrow chimeras (see below), all mice were age matched at 6–8 weeks old. *Il1r*$^{-/-}$ and C57BL/6 mice were purchased from Jackson Laboratories. *Casp1/11*$^{-/-}$ mice (32) were a gift from A. Van der Elden and M. Starnbach. *Il1a*$^{-/-}$ and *Il1b*$^{-/-}$ mice have been previously described (33). *Il1a/Casp1/11*$^{-/-}$ triple knockout mice were generated from crosses at UC Berkeley. B6.SJL-*Ptprc*^{a}/BoyAiTac (CD45.1) mice were purchased from Taconic. For bone marrow chimeras, 5–6 week old mice were irradiated twice with 600 rad 4 hours apart and reconstituted with 1×10^{7} donor cells by injection into the tail vein. Chimeric mice were bled 11 weeks after irradiation and reconstitution was assessed by flow cytometry of hematopoietic cells for expression of CD45.1 and CD45.2 using anti-CD45.1-

FITC (eBioscience) and anti-CD45.2-PE (eBioscience) antibodies. 12 weeks after irradiation chimeric mice were infected with *L. pneumophila*. All mice were specific pathogen free, maintained under a 12hr light-dark cycle (7 a.m. to 7 p.m.) and given a standard chow diet (Harlan irradiated laboratory animal diet) *ad libitum*.

In vivo experiments

Age matched mice were anesthetized with ketamine and infected intranasally with 2×10^6 LP01 or LP01Δ*dotA* in 20μL PBS. In some experiments mice were treated intranasally with ExoA, Pam3CSK4, or both in 20μL PBS, as described before (31). Bronchoalveolar lavage was performed by introducing 800μL of PBS into the trachea with a catheter (BD Angiocath 18g, 1.3648mm). Cells in the BAL fluid were pelleted and cell free BAL fluid was analyzed by ELISA. Total host cells in the lavage fluid were counted by staining cells with Guava Viacount (Millipore) and running samples on the Guava easyCyte Plus flow cytometer running CytoSoft5.3 software (Millipore). Lavage samples were stained with anti-Gr-1-PeCy7 and anti-Ly-6G-PE (eBioscience) and analyzed on a Beckman-Coulter FC-500 analyzer. Absolute numbers of Ly-$6G^+Gr1^+$ cells were calculated by taking the percent double positive cells determined by flow cytometry and multiplying by the total number of viable cells counted by the Guava easyCyte Plus flow cytometer. Bacterial burden in lungs was enumerated by hypotonic lysis of host cells in the lavage followed by spiral plating onto charcoal BYE plates with the Autoplate 5000 spiral plating system (Spiral Biotech, Inc.). CFU/mL in BAL fluid was determined by a QCount Colony Counter (version 3.0; Advanced Instruments, Inc.). BAL fluid mass was recorded prior to processing and this mass was used to estimate the volume of recovered BAL fluid. Total CFU was then calculated by multiplying CFU/mL by the estimated volume of BAL fluid. When noted, mouse body temperature and weight were monitored after infection with LP01. Mouse body temperature was measured by rectal probe and microtherma thermometer (Braintree Scientific). The probe was lubricated with a water-based lubricant (Astroglide) before use. Temperature and weight were measured at the same time daily.

Bacterial strains

For *in vitro* experiments all *L. pneumophila* strains were derived from LP02, a streptomycin-resistant thymidine auxotroph derived from *L. pneumophila* LP01. The Δ*dotA*, Δ*flaA*, Δ*5*Δ*flaA* strains were generated on the LP02 background and have been described previously (21, 30, 31). Mutants lacking one or more effectors were generated from LP02 by sequential in-frame deletion using the suicide plasmid pSR47S as described (34). Sequences of primers used for constructing deletion plasmids are listed in Supplemental Table I-A. Unless otherwise noted, all strains used for *in vitro* infections were deficient for bacterial flagellin (Δ*flaA*) and thus non-motile. *L. pneumophila* from the Δ*flaA* background were utilized *in vitro* to avoid activation of the NAIP5/NLRC4 inflammasome (19–23). For *in vivo* experiments, we utilized *L. pneumophila* wild-type strain LP01, a non-motile streptomycin-resistant strain derived from the original Philadelphia outbreak (35). The Δ*dotA* LP01 strain has been previously described (36).

Infection and stimulation

Bone marrow derived macrophages were plated in 24 well plates at a density of 5×10^5 cells per well and infected at an MOI of 1–3 (as indicated) by centrifugation for 10min at 400 ×*g*. In some experiments macrophages were treated with Exotoxin A (List Biological Labs), a synthetic bacterial lipopeptide (Pam3CSK4) (Invivogen), or both. After one hour of infection, media was changed. All *in vitro L. pneumophila* infections were in the absence of thymidine to curtail bacterial replication.

ELISA and Cytotoxicity

At the indicated time post-treatment, supernatants or bronchoalveolar lavage fluid were collected, cleared by centrifugation and analyzed by ELISA using paired interleukin-1α antibodies (BD Biosciences and eBioscience) or paired interleukin-1β antibodies (eBioscience and BD Bioscience). Recombinant IL-1α (eBioscience) or IL-1β (eBioscience) was used as a standard for each respective ELISA. Cytotoxicity was measured by evaluation of lactate dehydrogenase (LDH) released from cells (37). Specific lysis was calculated as a percentage of LDH released by detergent-lysed macrophages.

Cell culture

Macrophages were derived from the bone marrow of C57BL/6J mice (Jackson Laboratory). Macrophages were derived by 8 days of culture in RPMI supplemented with 10% serum, 100μM streptomycin, 100U/mL penicillin, 2mM L-glutamine and 10% supernatant from 3T3-macrophage-colony stimulating factor cells, with feeding on day 5. HEK293T cells were grown in complete media (DMEM, 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine).

Effector library screen

The library of 259 confirmed or putative secreted effector proteins has been previously described (38). Using the Gateway cloning system (Invitrogen) the library was cloned into a Gateway compatible MSCV 2.2 retroviral expression construct. We modified the MSCV 2.2 expression construct with an in-frame 6x-Myc tag upstream of the cloned effectors to accommodate for non AUG start codon usage in prokaryotes and we removed the downstream internal ribosome entry site (IRES)- green fluorescent protein (GFP). HEK293T cells were plated at 2.5×10^4 cells per well in 96-well tissue culture plates. 24hrs after plating cells were co-transfected using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions, with a single library clone and the TK-*Renilla* luciferase reporter construct. 24hrs after transfection cells were lysed in passive lysis buffer (Promega) for 5 min at 25°C. Cell lysates were incubated with the *Renilla* luciferase substrate coelentrerazine (Biotium) and luminescence was measured on a SpectraMax L microplate reader (Molecular Devices). The relative block in translation was measured by comparing *Renilla* luminescence in cells transfected with a control bacterial protein that does not block translation.

Quantitative RT-PCR

Macrophage RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA samples were treated with RQ1 DNase (Promega) prior to reverse transcription (RT) with Superscript III (Invitrogen). cDNA reactions were primed with poly(dT). Quantitative PCR was performed as described previously(39) using a Step One Plus RT-PCR system (Applied Biosystems) with Platinum *Taq* DNA polymerase (Invitrogen) and EvaGreen (Biotium). Transcript levels were normalized to those of *Rps17*. The following primer sequences were used: for *Il1a*, Forward 5'-ATGACCTGC AACAGGAAGTAAAA-3' and Reverse 5'-TGTGATGAGTTTTGGTGTTTCTG-3' and for *Rps17*, Forward 5'-CGCCATTATCCCCAGCAAG-3' and Reverse 5'-TGTCGGGATCCACCT CAATG-3'.

^{35}S-methionine metabolic labeling

5×10^5 bone marrow derived macrophages were seeded in 24-well plates and infected with bacterial strains at an MOI of 3. 25 minutes prior to labeling, macrophages were treated with 25μg/mL chloramphenicol to inhibit bacterial translation. At 6 and 24hrs post-infection media was removed and incubated with 25μCi/mL ^{35}S-methionine (Perkin Elmer) in RPMI

without methionine supplemented with 10% serum, 2mM L-glutamine, 25μg/mL and 10% supernatant from 3T3-macrophage-colony stimulating factor cells. Cells were labeled for 1 hour, washed three times with cold PBS and then lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with 2 mM Na_3VO_4, 1 mM PMSF, 25 mM NaF, and 1x Roche protease inhibitor cocktail (no EDTA), pH 7.2, for 10 minutes at 4°C. Total protein levels were measured by bicinchoninic acid (BCA) assay and equal amounts of protein were mixed with SDS sample buffer (40% glycerol, 8% SDS, 2% 2-mercaptoethanol, 40 mM EDTA, 0.05% bromophenol blue and 250 mM Tris-HCl, pH 6.8), boiled for 5 min and then separated by SDS–PAGE. The gels were stained with coomassie blue to show equal protein loading, dried, and exposed to a phosphor screen and visualized using a Typhoon Trio imager (GE Healthcare)

Results

Type-I IL-1 Receptor-deficient mice are more susceptible to *L. pneumophila* infection

A previous report identified the type-I interleukin-1 receptor (IL-1R) as a major signaling pathway that controls the recruitment of neutrophils to the lung in response to *L. pneumophila* (6). This report proposed that IL-1β is the major ligand signaling through the IL-1R in *L. pneumophila* infections, but did not specifically address a possible role of IL-1α and also did not examine the consequences of IL-1R deficiency on host health. Before addressing the relative importance of IL-1α and IL-1β, we first set out to confirm the previously proposed role of IL-1R signaling in *L. pneumophila* infection. We infected IL-1R-deficient (*Il1r*$^{-/-}$) mice with wild-type *L. pneumophila* and examined the mice at 12hrs, 24hrs and 48hrs post-infection. Total numbers of $Ly6G^+Gr1^+$ cells (here referred to as neutrophils) in bronchoalveolar lavage (BAL) fluid were determined by flow cytometry, and bacterial burden was measured by plating for colony forming units (CFUs). Consistent with previous reports, *Il1r*$^{-/-}$ mice recruited reduced numbers of neutrophils to the lungs in response to *L. pneumophila*, with approximately 10-fold, 5-fold and 4-fold fewer neutrophils in *Il1r*$^{-/-}$ mice than WT mice at 12hrs, 24hrs and 48hrs post-infection, respectively (Fig. 1A, B, C). Interestingly, while there are significant defects in the number of neutrophils recruited to the lungs of *Il1r*$^{-/-}$ mice, the total number of cells in the BAL fluid of these mice does not significantly differ from WT mice (Fig. 1A, B, C). The similarity in overall numbers of cells in the BAL appears to be because after infection with *L. pneumophila, Il1r*$^{-/-}$ mice harbor greater numbers of alveolar macrophages and CD45-negative/low cells that compensate for the decrease in neutrophils (Supplemental Fig. S1). One possible explanation for this is that in WT mice, damaged or dead alveolar macrophages and CD45-negative/low cells are normally phagocytosed and thereby eliminated by neutrophils. Thus, with decreased neutrophils in *Il1r*$^{-/-}$ mice, alveolar macrophages and CD45-negative/low cells accumulate (Supplemental Fig. S1). In addition to decreased neutrophils, *Il1r*$^{-/-}$ mice harbor approximately 5-fold and 17-fold higher CFU in BAL fluid over B6 controls at 24 and 48 hours post-infection, respectively (Fig. 1B, C). *Il1r*$^{-/-}$ mice also have a slight increase in bacterial burden measured in BAL fluid at 12hrs post-infection, but this difference is not dramatic, presumably because *L. pneumophila* does not have enough time to appreciably replicate or be cleared by the host at this time-point (Fig. 1A).

Although our data confirm that IL-1R signaling is critical for neutrophil recruitment and elimination of bacteria from the lung, neutrophils are also believed to be key mediators of the immune pathology of Legionnaires' Disease. Therefore we were interested to determine whether the decreased neutrophil response in *Il1r*$^{-/-}$ mice resulted in overall increased or decreased host health. To assay host health, we followed body temperature and weight loss in wild-type and *Il1r*$^{-/-}$ mice (Fig. 1D). Although both wild-type and *Il1r*$^{-/-}$ mice eventually recover from the infection, *Il1r*$^{-/-}$ mice show more severe weight loss and temperature

decreases than WT mice after being infected with *L. pneumophila* over a two week study (note that, in contrast to humans, mice typically exhibit a hypothermic response, rather than a fever, as a result of infection (40). We have shown that *Il1r*$^{-/-}$ mice exhibit increased bacterial burden in the lung at 12, 24 and 48 hours post-infection with *L. pneumophila* (Fig. 1A–D). We therefore suggest that increased bacterial burden in *Il1r*$^{-/-}$ mice over the first week of infection is likely the cause of the decreased overall health of these animals in response to *L. pneumophila* infection. However, after about a week of infection, compensatory innate and/or adaptive immune responses likely control the infection. Overall, our results suggest that during the course of experimental *L. pneumophila* infection, the beneficial function of early neutrophil influx in bacterial clearance outweighs the potentially negative effects of neutrophil-mediated immune pathology. These data also establish an important role for IL-1R signaling in host health in addition to the previously established role for IL-1R signaling in neutrophil recruitment and bacterial clearance.

IL-1α production precedes the recruitment of neutrophils to the lung

IL-1α and IL-1β are the only known agonists of the IL-1R. We therefore tested whether there was a correlation between IL-1α or IL-1β production and the recruitment of neutrophils to the lungs of infected mice. B6 mice were infected with wild-type *L. pneumophila* or an avirulent mutant strain of *L. pneumophila* that lacks a functional Type IV secretion system (Δ*dotA*). BAL fluid was harvested at 3, 6, 9 and 12 hours post-infection, and assessed for the presence of neutrophils, IL-1α, and IL-1β. The earliest *in vivo* Dot/Icm-dependent response was the production of IL-1α, which was first detectable at 3hrs post-infection (Fig. 2A). By contrast, the earliest significant production of IL-1β (above that induced by Δ*dotA*) was not until 6hrs post-infection (Fig. 2B), the same time that the Dot/Icm-dependent influx of neutrophils can first be detected (Fig. 2C). It is interesting to note that there seems to be an increase in the number of neutrophils found in the BAL fluid after infection with the Δ*dotA L. pneumophila* strain at 3hrs post-infection, although this difference is not statistically significant (Fig. 2C). Consistent with previous results, the Δ*dotA L. pneumophila* strain did not appreciably induce IL-1α production in the lung (Fig. 2A, B). Thus, while there may be a low level of Dot-independent neutrophil recruitment to the lung, this recruitment appears to be IL-1α independent and likely plays a minimal role in protecting the host from infection. Taken together, these data show that IL-1α production is largely Dot-dependent and occurs prior to the recruitment of neutrophils to the lung. Our findings suggest a role for IL-1α in the early IL-1R-dependent and Dot-dependent recruitment of neutrophils to the lungs of *L. pneumophila* infected mice.

IL-1α, but not IL-1β, is required for early neutrophil recruitment to the lung

We next tested whether the loss of IL-1α or IL-1β would have an effect on neutrophil recruitment. We infected wild-type (B6), *Il1a*$^{-/-}$, *Il1b*$^{-/-}$, *Casp1/11*$^{-/-}$ and *Il1r*$^{-/-}$ mice with wild-type *L. pneumophila* and measured neutrophil recruitment and bacterial burden at 12hrs post-infection. As expected, *Il1r*$^{-/-}$ mice showed a strong defect in recruitment of neutrophils to the lung, while both the *Il1b*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice showed no defect in neutrophil recruitment to the lung at 12hrs post-infection, as compared to B6 mice (Fig. 3A). However, there was approximately a 17-fold decrease in the number of neutrophils recruited to the lung of *Il1a*$^{-/-}$ mice as compared to B6 mice (Fig. 3A). Importantly, *Il1a*$^{-/-}$ mice have no significant difference in the production of IL-1β in the BAL fluid of infected mice at 12hrs post-infection (Supplemental Fig. S2A, B). These data suggest that IL-1α may be more important than IL-1β for the recruitment of neutrophils to the lung at 12hrs post-infection. Interestingly, the defect in neutrophil recruitment in *Il1a*$^{-/-}$ mice was not as pronounced as the defect seen in *Il1r*$^{-/-}$ mice. This suggests that although IL-1β is not itself essential for neutrophil recruitment, it can partially compensate for the loss of IL-1α (addressed further below). The bacterial burden in the infected mice was very similar among

all of the genotypes, likely because at 12hrs post-infection *L. pneumophila* has not had enough time to appreciably grow or be cleared by the host immune response (Fig. 3B). As an important control, measurement of IL-1α protein levels in the BAL fluid of infected mice demonstrated that only *Il1a*$^{-/-}$ mice had defects in production of IL-1α in response to *L. pneumophila* infection (Fig. 3C). The amount of IL-1α detected in the BAL fluid of *L. pneumophila* infected *Il1r*$^{-/-}$ mice is slightly higher than WT mice, likely due to an increase in bacterial burden caused by reduced neutrophil recruitment to the lungs of these mice (Fig. 3C). The increase in bacterial burden in *Il1r*$^{-/-}$ mice likely leads to more infected macrophages and thus an increase in the production of IL-1α. Additionally, the loss of the IL-1R may result in less internalization of the IL-1α protein, resulting in higher extracellular accumulation. We also note that IL-1α is produced even in *Casp1/11*$^{-/-}$ mice, indicating that in response to *L. pneumophila* IL-1α production *in vivo* can be independent of both Caspase-1 and Caspase-11 inflammasomes (Fig. 3C).

We hypothesized that the intermediate phenotype seen in the *Il1a*$^{-/-}$ mice was due to low levels of inflammasome-dependent IL-1β production that are still capable of signaling through the IL-1R. We were unable to generate *Il1a/b*$^{-/-}$ double knockout mice as these genes are located directly next to each other on the chromosome. Thus, to test whether there is redundancy between IL-1α and IL-1β, we generated *Il1a/Casp1/11*$^{-/-}$ 'triple' knockout mice (TKO). These mice are predicted to be deficient in production of IL-1α and IL-1β, as production of biologically active IL-1β is generally believed to require Caspase-1. We should note that the TKO mice are not only defective in IL-1β cytokine production, but they are also unable to undergo pyroptosis, a Caspase-1/11-dependent form of lytic cell death, which has previously been shown to evict bacteria from their intracellular niche and render them susceptible to phagocytosis and killing by neutrophils (41, 42). The loss of pyroptosis could lead to an increased bacterial burden; however, at 12hrs post-infection we see very little differences in bacterial burden in the BAL fluid and thus we argue that the major defect in the TKO mice at 12hrs post-infection is the loss of IL-1β processing and release (Fig. 3D, E). Consistent with a defect in IL-1α and IL-1β production, we find that in response to *L. pneumophila* infection TKO mice produce almost no detectable IL-1α and very low levels of IL-1β in BAL fluid at 12 hours post-infection (Supplemental Fig. S2C, D). Interestingly, TKO mice exhibited a large defect in neutrophil recruitment to the lung; in fact, these mice were as defective in neutrophil recruitment as *Il1r*$^{-/-}$ mice (Fig. 3D). These data suggest that IL-1α is the major cytokine required to signal through the IL-1R and recruit neutrophils to the lung at 12 hours post-infection, though Casp1/11-dependent signaling through the IL-1R (presumably mediated by IL-1β) can partially compensate for the loss of IL-1α. Furthermore, Caspase-1 is usually considered to be essential for IL-1β processing (15, 16), although some previous reports have suggested that IL-1β can be generated in the absence of *Casp1/11* (43, 44). Even though *Il1a/Casp1/11*$^{-/-}$ TKO mice produced very low levels of IL-1β, TKO mice were as defective in neutrophil recruitment as *Il1r*$^{-/-}$ mice, implying that, at least in response to *L. pneumophila*, production of biologically active IL-1β requires Caspase-1/11.

At late timepoints after infection, IL-1β compensates for the loss of IL-1α

Il1r$^{-/-}$ mice exhibit reduced neutrophil recruitment that is sustained until at least 48hrs post-infection (Fig. 1A). We therefore tested if the loss of IL-1α would lead to a defect in neutrophil recruitment and an increase in bacterial burden at late timepoints. We infected wild-type, *Il1a*$^{+/-}$, and *Il1a*$^{-/-}$ littermates with wild-type *L. pneumophila* and compared these mice to *Casp1/11*$^{-/-}$ mice (Fig. 3F–H). At 48hrs post-infection *Il1a*$^{-/-}$ mice have no defect in neutrophil recruitment to the lung and only a modest defect in control of bacterial burden (Fig. 3F–G). Additionally we see no defect in neutrophil recruitment by *Casp1/11*$^{-/-}$ mice at 48hrs post-infection, suggesting that both IL-1α and IL-1β are capable of signaling

through the IL-1R and can compensate for the loss of each other by 48h post-infection. In fact, *Casp1/11*$^{-/-}$ mice actually appeared to exhibit increased recruitment of neutrophils to the lung (Fig. 3F). However, despite the increased neutrophil recruitment, *Casp1/11*$^{-/-}$ mice also exhibited increased bacterial burdens in the lung at 48h post-infection (Fig. 3G). As mentioned previously, this counterintuitive result is likely explained by the loss of Caspase-1/11-dependent pyroptosis, which has previously been shown to evict bacteria from their intracellular niche and render them susceptible to phagocytosis and killing by neutrophils (41, 42). Importantly, we find that *Casp1/11*$^{-/-}$ mice produce IL-1α in response to *L. pneumophila* infection and actually induce significantly more IL-1α than wild-type mice; this increase is likely due to the loss of pyroptosis and subsequent increased bacterial burden in these mice (Fig. 3H).

IL-1α is produced by cells derived from the hematopoietic lineage

IL-1α is inducible in hematopoietic cells, but is also reported to be constitutively expressed by certain non-hematopoietic cells (24, 28). We therefore wished to determine whether the rapid production of IL-1α and the ensuing neutrophil influx required IL-1α production by hematopoietic or non-hematopoietic cells. We generated bone marrow chimeras in which wild-type B6 (CD45.1^{+}) mice were reconstituted with bone marrow from *Il1a*$^{-/-}$ (CD45.2^{+}) mice, and *vice-versa*. To confirm that our chimeras had been reconstituted to a high level, blood samples were collected and stained with antibodies for CD45.1 and CD45.2 that marked wild-type and *Il1a*$^{-/-}$ derived hematopoietic cells, respectively (Supplemental Fig. S3). Chimeric mice were infected with *L. pneumophila* and BAL fluid was collected 12 hours post-infection. Mice reconstituted with B6 hematopoietic cell populations produced IL-1α in response to *L. pneumophila* infection, whereas mice reconstituted with *Il1a*$^{-/-}$ bone marrow failed to produce IL-1α (Fig. 4A). Importantly, the production of IL-1α correlated with the recruitment of neutrophils to the lung (Fig. 4B). Consistent with our previous findings (Fig. 3) we see little difference in the total CFU found in the BAL fluid of these mice at 12h post-infection, although there was a slight increase in bacterial burden in mice that received *Il1a*$^{-/-}$ bone marrow (Fig. 4C). These chimera experiments demonstrate that hematopoietic cells in the lung, presumably macrophages that have been infected with *L. pneumophila*, are responsible for the early production of IL-1α and subsequent recruitment of neutrophils to the site of infection.

L. pneumophila lacking effectors that block host protein synthesis still induce IL-1α

Given the major role IL-1α plays in neutrophil recruitment, we next wanted to explore the molecular mechanism of IL-1α production by macrophages. We (31) and others (34, 45, 46) previously showed that *L. pneumophila* encodes five Dot/Icm-secreted effectors that inhibit host protein synthesis. A strain lacking these five effectors (Δ*5*) was defective in the induction of a subset of inflammatory cytokines, including IL-23 and GM-CSF (31). Moreover, Δ*5* was also defective in the transcriptional induction of the *Il1a* gene when the Toll-like receptor (TLR) and NOD-like receptor innate immune sensing pathways were severely hindered (infections of *Myd88/Nod1/Nod2*$^{-/-}$ BMDMs)(30). The overall model emerging from our previous studies was that protein synthesis inhibition by virulent *L. pneumophila* produces a host cell stress response that leads to the production of inflammatory cytokines. Therefore, we asked whether the Δ*5 L. pneumophila* strain could still induce IL-1α protein release by wild-type BMDMs. We infected macrophages with the Δ*5 L. pneumophila* strain on the Δ*flaA* background (Δ*5*Δ*flaA*) strain and measured the production of IL-1α from these cells. We utilized *L. pneumophila* on the Δ*flaA* background to avoid the confounding effects of NAIP5/NLRC4 inflammasome activation by flagellin. Interestingly, we found that the Δ*5*Δ*flaA* strain still induced production of significant amounts of IL-1α protein (Fig. 5A). This result is consistent with previous *in vivo* observations that showed that neutrophil recruitment is normal in response to the Δ*5* mutant

(30). We considered two possible explanations for the ability of the Δ*5* mutant to induce IL-1α: (1) protein synthesis inhibition is not required for IL-1α production; or (2) residual protein synthesis inhibition by the Δ*5* strain is sufficient to induce IL-1α. Consistent with the latter possibility, and with our previous work (31), we found that the Δ*5*Δ*flaA* strain still significantly inhibited host protein synthesis in BMDMs (as measured by incorporation of ^{35}S-methionine) as compared to infection with Δ*dotA*Δ*flaA*, which does not block translation (Fig. 5B; 31). These results raised the possibility that *L. pneumophila* might encode additional effectors that inhibit host protein synthesis. To identify these effectors we utilized a library of 259 known and putative secreted effectors (38), that we cloned into a mammalian expression vector. Each individual effector expression plasmid was co-transfected into 293T cells, along with a plasmid that constitutively expresses *Renilla* luciferase, and protein synthesis (as assessed by luminescence) was measured 24hrs after transfection (Supplemental Fig. S3; Supplemental Table I-B). As a positive control, this screen successfully identified the five previously described effectors that are known to block host translation (Lpg0437, Lpg1368, Lpg1488, Lpg2504, and Lpg2862) (31; Supplemental Table I-B). In addition, two other effectors that inhibit host protein synthesis were identified: Lpg0208, a Serine/Threonine Kinase, and Lpg1489, a putative effector of unknown function (47). Lpg0208 and Lpg1489 were confirmed to inhibit protein synthesis in 293T cells, as measured by reduced ^{35}S-methionine incorporation upon overexpression of each effector (data not shown). However, deletion of these two additional effectors in the Δ*5*Δ*flaA* background, to generate a strain we call Δ*7*Δ*flaA*, did not significantly affect the ability of *L. pneumophila* to inhibit host protein synthesis in macrophages (Fig. 5B). The Δ*7* strain also induced normal production of IL-1α *in vitro* (Fig. 5A). The residual ability of Δ*7 L. pneumophila* to inhibit host protein synthesis and/or induce IL-1α may therefore be due to additional effectors that were not present in our effector library. Alternatively, inhibition of host protein synthesis may result from the combined effects of multiple *L. pneumophila* effectors (which would not have been detected in our one-by-one effector screen), or the infection process itself.

Translation inhibition together with TLR activation is sufficient to induce IL-1α production *in vitro* and *in vivo*

The above results showed that induction of IL-1α by Δ*7 L. pneumophila* correlates with inhibition of host protein synthesis. We therefore wished to determine if inhibition of host protein synthesis is sufficient to cause IL-1α release *in vitro* and *in vivo*. In order to recapitulate TLR signaling that occurs during *L. pneumophila* infection, bone marrow derived macrophages (BMDMs) were treated with 10ng/mL Pam3CSK4, a TLR2 ligand. This treatment induced transient intracellular IL-1α protein (Fig. 6A) but did not result in significant IL-1αrelease (Fig. 6B). BMDMs were therefore additionally treated with 50ng/mL Exotoxin A (ExoA), a toxin made by *Pseudomonas aeruginosa* that blocks translation by inhibiting the activity of elongation factor 2a (reviewed in 48, 49). As with TLR stimulation, ExoA treatment alone was insufficient to induce IL-1α production. However, we found that treatment of BMDMs with both Pam3CSK4 and ExoA combined to induce release of IL-1α at 24h post-infection (Fig. 6A, B). ExoA appeared to have two important effects that might explain its role in IL-1α release. First, in contrast to the transient induction of IL-1α induced by TLR signaling alone, additional treatment with ExoA caused the sustained presence of intracellular IL-1α protein at 24h post-treatment, similar to what is seen in *L. pneumophila* infection (Fig. 6A). The sustained production of IL-1α protein was associated with a prolonged elevation of *Il1a* mRNA (Fig. 6C). Second, ExoA caused cell death by 24hrs post-treatment (Fig. 6D), which may explain how intracellular accumulated IL-1α is released from these macrophages. Δ*flaA* and Δ*5*Δ*flaA L. pneumophila*-infected macrophages, which also experience a block in host protein synthesis, show sustained transcriptional induction, release IL-1α from the cell, and undergo cell death at 24h post-

infection (Fig. 6A–D). Importantly, death of *L. pneumophila* infected cells does not appear to depend on bacterial replication because cell death and IL-1α release still occurred when bacterial replication was curtailed by removal of thymidine from the media. We speculate that inhibition of protein synthesis may be responsible for induction of host cell death. Protein synthesis inhibition and TLR stimulation also synergized to induce elevated IL-1α production *in vivo* (Fig. 6E). Taken together, these findings suggest that TLR activation in concert with translation inhibition can recapitulate IL-1α production and release in response to *L. pneumophila* infection and that this treatment is sufficient to induce release of IL-1α.

Discussion

Legionnaires' disease is an inflammatory pneumonia associated with a pronounced influx of neutrophils to the lung (3, 5). The recruitment of neutrophils to the lung is important for controlling bacterial burden; however, excessive neutrophil recruitment can also be detrimental to the host and may be responsible for immune pathologies associated with Legionnaires' disease (3, 5). Thus, the host must tightly regulate the recruitment of neutrophils to the site of infection. In animal models of *L. pneumophila* infection, neutrophil recruitment has been shown to be important for protecting the host (6, 8, 9), yet the mechanism for this recruitment has remained unclear. A number of studies have demonstrated that MyD88 is an important host factor that protects mice from *L. pneumophila* infection (6, 10–13) and the IL-1R has been shown to be the critical receptor upstream of MyD88 that controls the recruitment of neutrophils to the lung in response to *L. pneumophila* infection (6). Indeed, it has been shown that IL-1R signaling is required in AECs to induce chemokines, such as CXCL1 and CXCL2, which then recruit neutrophils to the site of infection (6).

In our study, we identify the cytokine interleukin-1α (IL-1α) as a critical initiator of IL-1R-dependent neutrophil recruitment to the lungs of *L. pneumophila*-infected mice. We find that IL-1α, but not IL-1β, precedes neutrophil recruitment to the lung and we show that IL-1α is generated specifically by cells in the hematopoietic compartment (presumably infected macrophages). Given these data, we therefore propose a model by which IL-1α is produced by alveolar macrophages in response to virulent *L. pneumophila* and signals through the IL-1R on AECs, amplifying the original signal and generating chemokines which recruit the initial wave of neutrophils to the lung. Importantly, at timepoints later than 12hrs post-infection, IL-1α and IL-1β can both signal through the IL-1R and compensate for the loss of each other. Our data suggest that IL-1α is one of the earliest cytokines produced in response to *L. pneumophila in vivo*, and thus initiates the recruitment of neutrophils and the inflammatory response to *L. pneumophila in vivo*.

Similar to *L. pneumophila*, *Streptococcus pneumoniae* leads to a severe pneumonia associated with massive influx of neutrophils. In mouse models of *S. pneumoniae* infection in the lung, *Il1a/I1b*$^{-/-}$ double knockout and *Il1b*$^{-/-}$ mice are more susceptible to disease and have decreased clearance of bacteria from the lung (50). Moreover, *Il1r*$^{-/-}$ mice have increased bacterial burden in the lung and decreased neutrophil recruitment to the lung (51). Macrophage uptake of *S. pneumoniae* induces inflammasome activation and IL-1β release which can signal to epithelial cells to recruit neutrophils by releasing the chemokine CXCL8 (51). Studies with *S. pneumoniae* suggest a model whereby activated macrophages secrete IL-1β which signals through the IL-1R of AECs thus leading to the production of chemokines, which recruit neutrophils to the site of infection (50, 51). This proposed mechanism is similar to the mechanism that we propose for *L. pneumophila* infections, except that it appears that IL-1α, rather than IL-1β, is the dominant cytokine early during *L. pneumophila* infections. These studies with *S. pneumoniae* suggest that amplification of early responses to infection by IL-1R signaling in AECs may be a conserved immune

strategy important for recruiting neutrophils in response to bacterial infections. Importantly, the role for IL-1α in *S. pneumoniae* infections remains unclear.

In addition to *S. pneumoniae*, IL-1R signaling has been shown to be important for host protection from numerous pathogens, including *Listeria monocytogenes* (52–54), *Mycobacterium tuberculosis* (36, 55–57), *Pseudomonas aeruginosa* (58), *Staphylococcus aureus* (59), *Klebsiella pneumoniae* (60) and *Candida albicans* (61). In many of these infections, the mechanism by which IL-1R provides protection is not clear, and the relative roles of IL-1α and IL-1β have not been elucidated. One study that dissected the relative roles of IL-1α and IL-1β during *M. tuberculosis* infection found each cytokine played essential and non-redundant roles *in vivo* (62). This study, along with our results showing that IL-1α is of primary importance in early responses to *L. pneumophila in vivo*, suggest it will be worthwhile to examine more carefully the relative contributions of IL-1α and IL-1β in mediating IL-1R-dependent responses to other pathogens as well.

The molecular mechanism leading to IL-1α production has remained elusive (28). This is in stark contrast to IL-1β production, where intensive effort over the past decade has defined the mechanisms leading to IL-1β release downstream of inflammasome activation (reviewed in 18). Our data suggest that equal attention should be paid to the mechanisms of IL-1α production. Indeed, IL-1α has been shown to be induced in response to a number of bacterial pathogens including *L. pneumophila* (29, 30), *L. monocytogenes* (63), *S. aureus* (64), and *M. tuberculosis* (57, 62); however, the molecular mechanism of IL-1α production in response to these pathogens remains unsettled. Classic studies showed that IL-1α can be cleaved by the Calpain family of calcium dependent proteases, but IL-1α does not appear to require processing to signal through the IL-1R (24, 25, 28). Some reports have suggested that IL-1α production in response to non-infectious stimuli such as toxins can involve activation of the Caspase-1 or Caspase-11 inflammasomes (24, 26, 27, 65). Additionally, a previous report suggests that at 4hrs post-infection *Casp1/11*$^{-/-}$ mice have defects in IL-1α production in response to *L. pneumophila* infection *in vivo* (27). In contrast to these reports, our data show that *Casp1/11*$^{-/-}$ mice have no defect in IL-1α production in response to *L. pneumophila* infection *in vivo*. This difference may be due to the different strains of *L. pneumophila* used in the two studies. Nevertheless, our results indicate that IL-1α and IL-1β can be produced via distinct but complementary pathways that provide alternative means to induce IL-1R signaling and immune defense *in vivo*. Given the critical importance of neutrophils in providing defense against numerous bacterial pathogens, it is perhaps to be expected that hosts would not rely on a single mechanism for activation of IL-1R signaling that could then be easily subverted or avoided.

Instead of a role for the inflammasome in IL-1α release, our data show that translation inhibition in concert with TLR stimulation is sufficient to induce IL-1α both *in vitro* and *in vivo*. Recent work from our lab and others have shown that in mice, and in *C. elegans*, translation inhibition can be sensed by the host and induce a number of immunological responses, including the production of pro-inflammatory cytokines (2, 30, 31, 66, 67). Previous research identified five *L. pneumophila* effectors that block host translation (31, 34, 45, 46). We previously found that this translation block induces a host stress response that can induce a subset of inflammatory cytokines, including IL-23 and GM-CSF (30, 31). Although the *L. pneumophila* Δ*5* strain lacking the five effectors is partially defective in its ability to inhibit host protein synthesis (31) and is defective for IL-23 and GM-CSF induction, we confirmed here that cells infected with Δ*5 L. pneumophila* still experience a significant block in protein synthesis. Consistent with our finding that protein synthesis inhibition and TLR signaling is sufficient to induce IL-1α, we also find that Δ*5*-infected cells still produce IL-1α. In fact, even after identifying two novel bacterial effectors that block host translation, and generating an *L. pneumophila* mutant (Δ*7*) that lacks these

effectors in addition to the original five effectors, we were still unable to abolish the Dot/Icm-dependent ability of *L. pneumophila* to inhibit protein synthesis and induce IL-1α. We propose several hypotheses to explain these results. First, there may be additional bacterial effectors in *L. pneumophila* that are not in our library of cloned effectors. Given that *L. pneumophila* is a generalist and has a multitude of natural hosts (68), it is possible that there is substantial additional redundancy encoded in the *L. pneumophila* genome. A second possibility is that there may not be a specific *L. pneumophila* effector that targets the host protein synthesis machinery; instead, the blockade of protein synthesis we observe may be the result of a *host* response to the infection process itself. Indeed, translation inhibition has long been recognized as a protective response during viral infections (69), and it is now evident that numerous bacterial infections can elicit host stress pathways that affect protein synthesis, for example via phosphorylation of eukaryotic initiation factor 2α (eIF2α) (69). Lastly, it is possible that the ability of *L. pneumophila* to induce IL-1α is unrelated to protein synthesis inhibition. We tend not to favor this latter possibility because we found that inhibition of protein synthesis in conjunction with TLR signaling was sufficient to induce IL-1α, and moreover, it is clear that *L. pneumophila* infection results in both TLR signaling and inhibition of protein synthesis. Thus, while the mechanism of IL-1α production continues to elude the field, it seems likely that translation inhibition is at least one mechanism for IL-1α induction, even if other parallel mechanisms might also exist.

Together, our data show that IL-1α is a major cytokine responsible for the early recruitment of neutrophils to the lung in response to *L. pneumophila* infection from 0–12 hours post-infection. We propose that a dominant role for IL-1α in protection against microbial infection may hold true for other pathogens, depending on the stage and mode of infection. Although much recent work has focused on the mechanisms of IL-1β production, our work suggests that IL-1α signaling can be as important, or indeed more important, than IL-1β signaling *in vivo*. Indeed, it is probably evolutionarily advantageous for hosts to encode multiple parallel pathways to induce IL-1R signaling, given the critical role that the IL-1R appears to play in orchestrating neutrophil recruitment and other immune responses *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

T4SS Type-IV Secretion System

IL-1R	Interleukin-1 Receptor Type I
AECs	airway epithelial cells
BAL	bronchoalveolar lavage
TKO	triple knockout
BMDM	bone marrow derived macrophage
ExoA	Exotoxin A

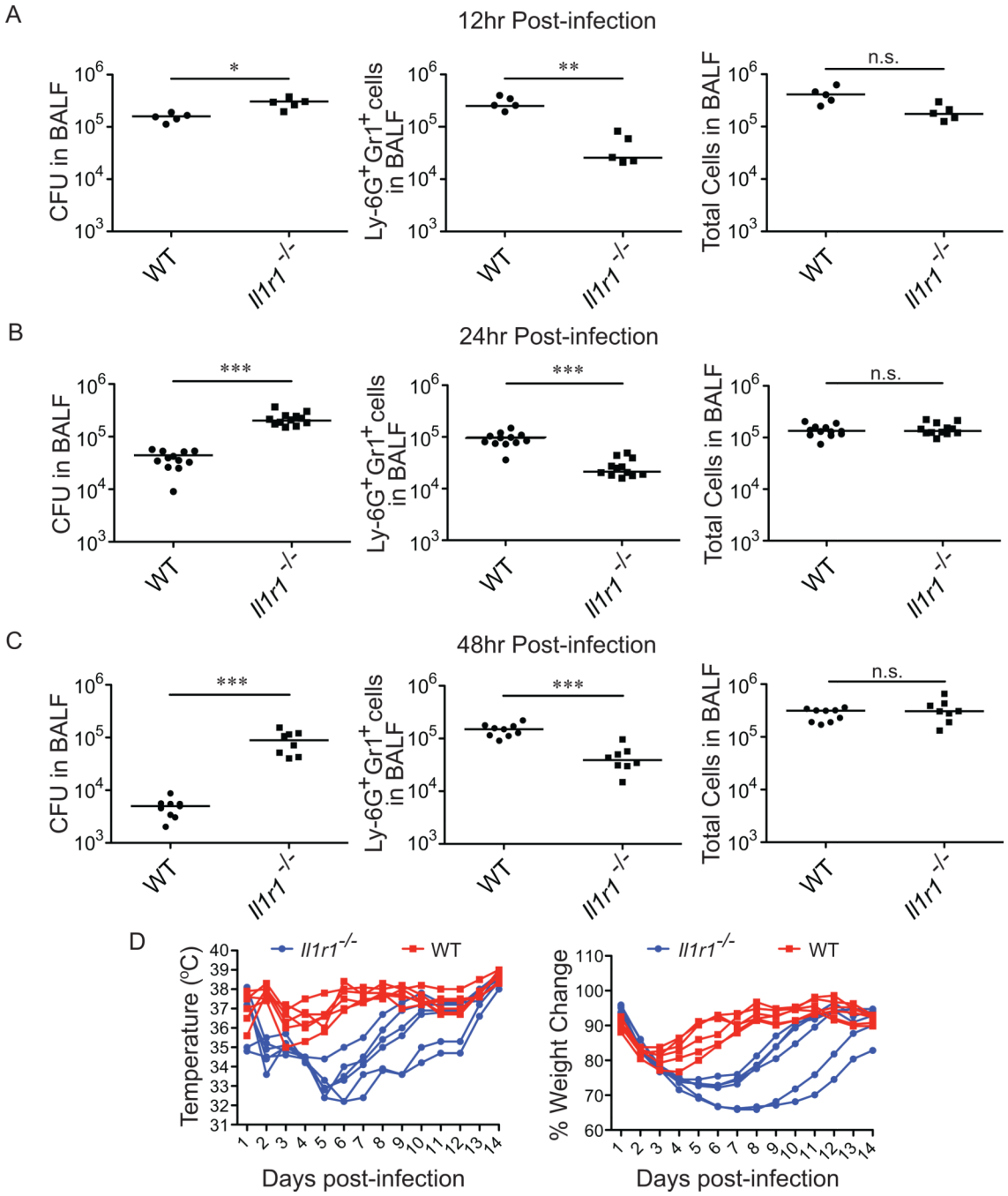

Figure 1.

The IL-1 Receptor Type I is essential for control of *L. pneumophila* infection. (A–C) IL-1R Type I-deficient mice were infected intranasally with 2×10^6 *L. pneumophila* (LP01). Bronchoalveolar lavage (BAL) was performed at12 hours (A), 24 hours (B) and 48 hours (C) post-infection. Bacterial burden in the BAL fluid was determined by plating for colony forming units. The number of Ly-$6G^+Gr1^+$ cells was determined by flow cytometry and the total number of cells in the BAL fluid as determined by Guava ViaCount assay. (D) IL-1R-deficient (blue circles) and wild-type B6 (red squares) mice were infected with 2×10^6 *L. pneumophila* (LP01) and monitored daily for temperature and weight change. Percent weight change is calculated to weight at day zero. Data are representative of two (D) or three

(A, B, C) experiments. (Median in A-C). *, $p<0.05$. **, $p<0.01$. ***, $p<0.005$. (Statistical analysis: Mann-Whitney U test).

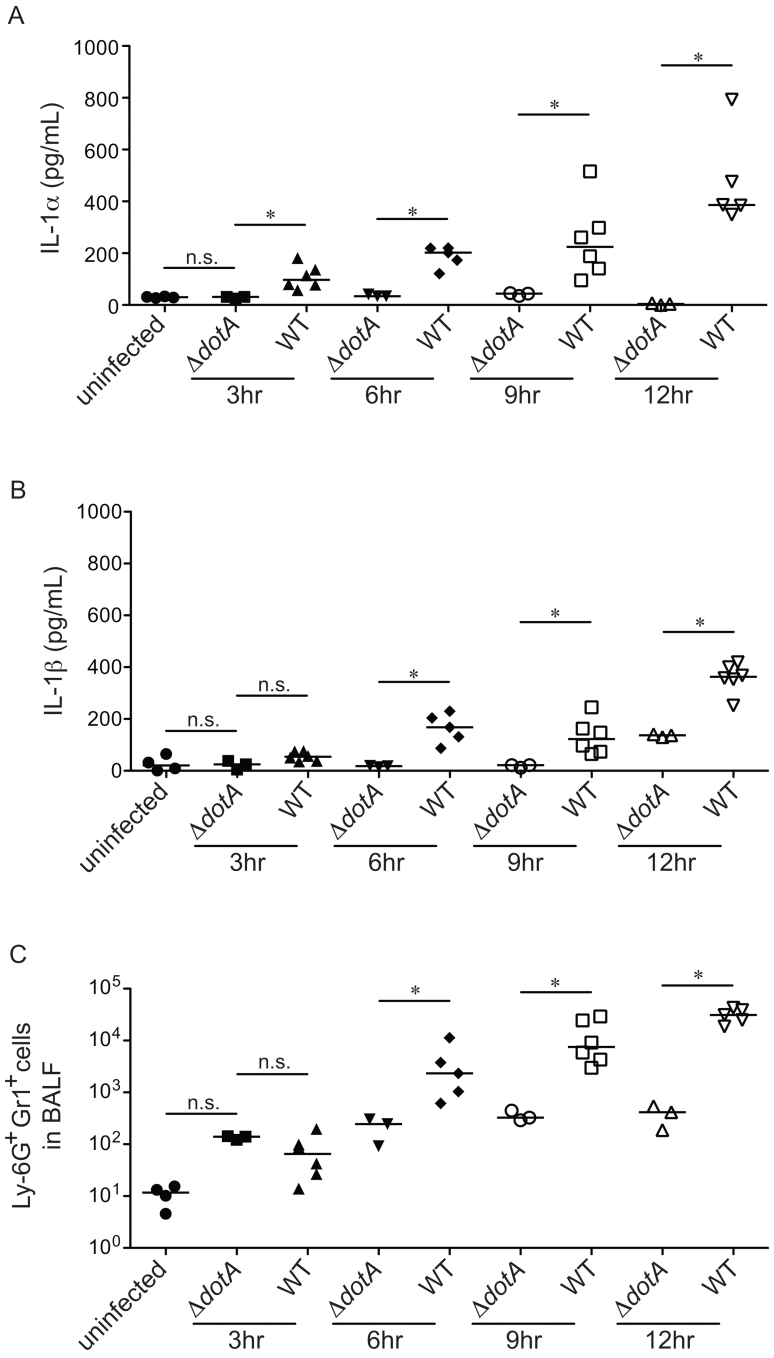

Figure 2.

Dot/Icm T4SS-dependent IL-1α production precedes the recruitment of Ly-6G$^+$Gr1$^+$ cells to the lung. (A–C) B6 mice were infected intranasally with 2×10^6 *L. pneumophila* (WT) or a mutant lacking a function type IV secretion system (Δ*dotA*). Bronchoalveolar lavage was performed at 3, 6, 9, or 12 hours post-infection. IL-1α (A) and IL-1β (B) levels were measured by ELISA. (C) The number of Ly-6G$^+$Gr1$^+$ positive cells in the BAL fluid was determined by flow cytometry. Data are representative of three experiments. (Median in A-C). *, $p<0.05$ (Statistical analysis: Mann-Whitney U test).

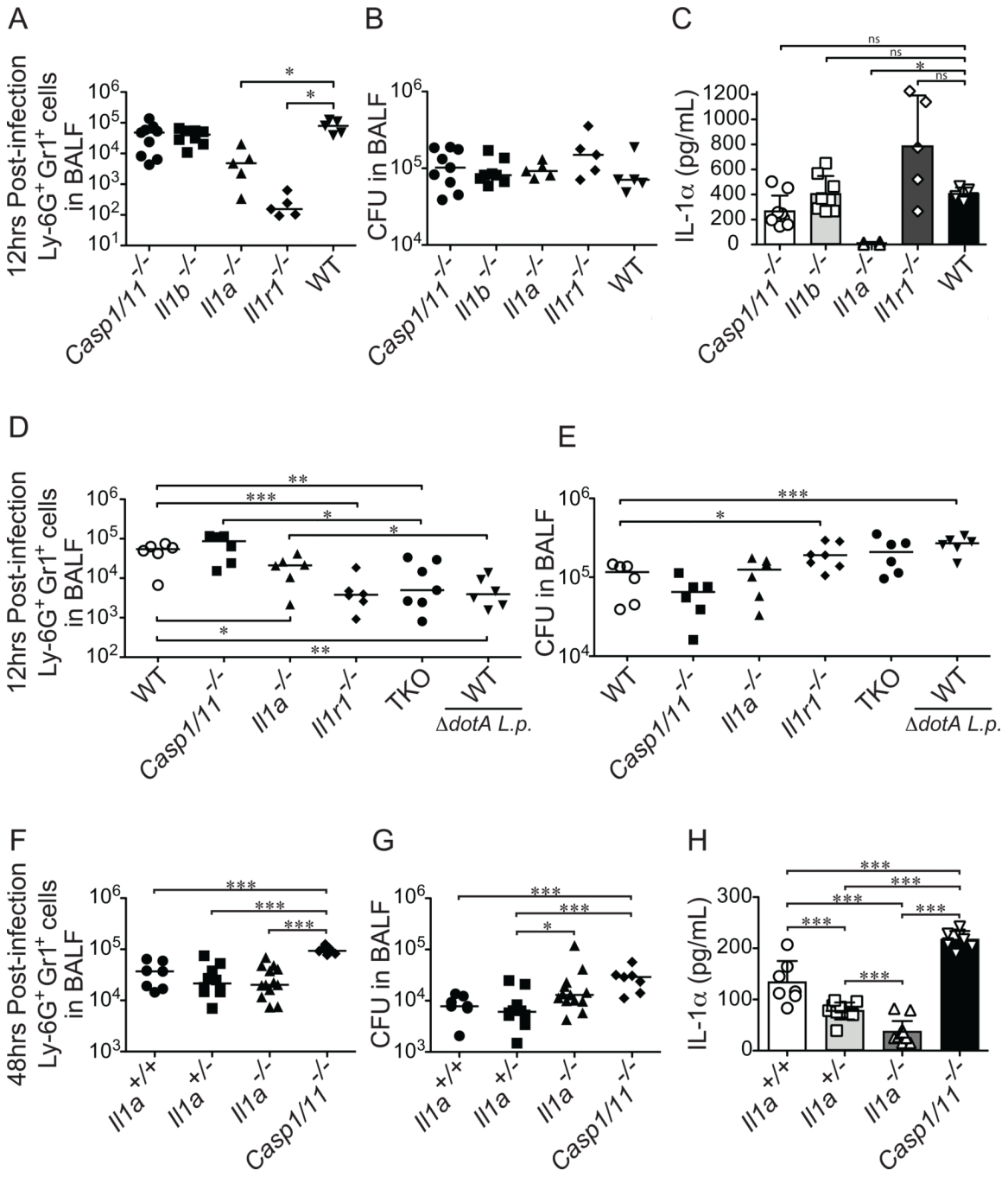

Figure 3.

IL-1α is required for Ly-6G$^+$Gr1$^+$ cell recruitment to the lung in response to infection with *L. pneumophila*. (A–C) The indicated mouse strains were infected intranasally with 2×10^6 *L. pneumophila* (LP01). At 12hrs post-infection bronchoalveolar lavage (BAL) fluid was collected. (A) Ly-6G$^+$Gr1$^+$ cells in the BAL fluid were enumerated by flow cytometry. (B) Bacterial burden in the lung was determined by plating BAL fluid for CFU. (C) IL-1α levels were measured by ELISA. (D–E) The indicated mouse strains were infected intranasally with 2×10^6 *L. pneumophila* (LP01) or Dot/Icm T4SS-deficient *L. pneumophila* (LP01 Δ*dotA*) as noted. At 12 hours post-infection BAL fluid was harvested and Ly-6G$^+$Gr1$^+$ cell recruitment was measured by flow cytometry (D) and bacterial burden in the lung was

measured by plating for CFU (E). (F–H). *Il1a*$^{-/-}$*Il1a*$^{+/-}$, and *Il1a*$^{+/+}$ littermates were infected intranasally with 2×10^6 *L. pneumophila* (LP01). Non-littermate *Casp1/11*$^{-/-}$ mice were also infected with LP01. Bronchoalveolar lavage fluid was collected 48hrs post-infection and Ly-6G$^+$Gr1$^+$ cells were quantified by flow cytometry (F). Bacterial burden was determined by plating for CFU (G). IL-1α levels in the BAL fluid were determined by ELISA (H). Data are representative of two (F–H) or three (A–E) experiments (Median in A, B, D-G. mean ± s.d. in C, H). The low level of apparent IL-1α protein produced in *Il1a*$^{-/-}$ mice at 48h post infection appears to be due to an unknown cross-reacting protein that produced a low signal on the ELISA assay. TKO, *Il1a/Casp1/11*$^{-/-}$ triple knockout mice. *, $p<0.05$. **, $p<0.01$. ***, $p<0.005$. (Statistical analysis: Mann-Whitney U test).

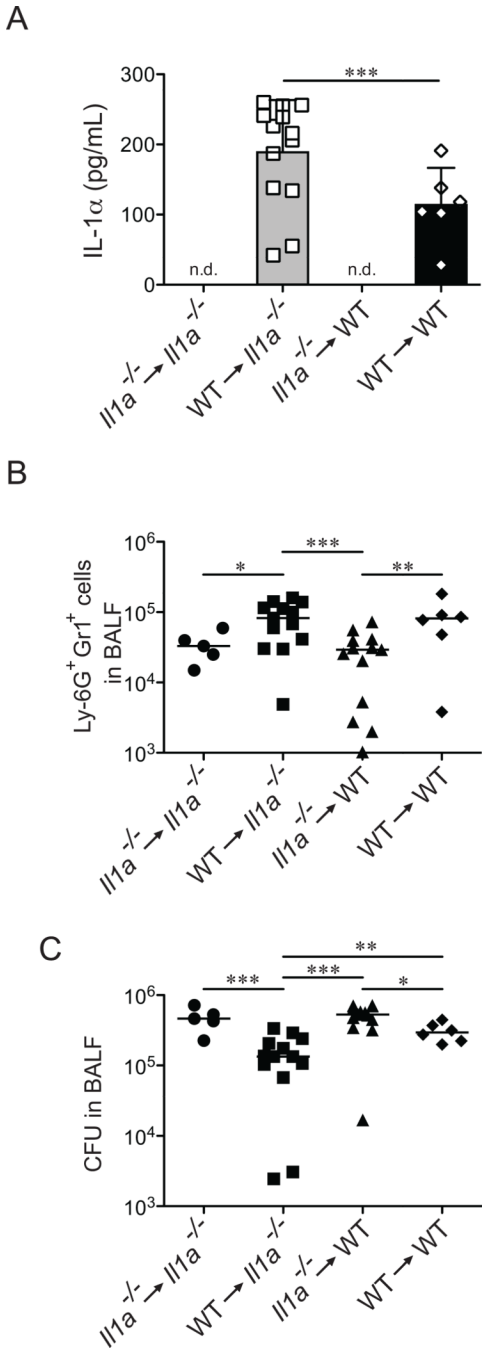

Figure 4.

Hematopoietic cells are responsible for IL-1α production in response to *L. pneumophila*. (A–C) 6 week old *Il1a*$^{-/-}$ and congenically marked B6.SJL (CD45.1) mice were lethally irradiated and reconstituted with *Il1a*$^{-/-}$ (CD45.2) or B6.SJL bone marrow as indicated. After 12 weeks of recovery, chimeric mice were infected with *L. pneumophila* (LP01). Bronchoalveolar lavage (BAL) fluid was collected 12hrs post-infection. (A) IL-1α levels in BAL fluid were determined by ELISA. (B) Recruitment of Ly-6G^{+}Gr1^{+} cells was determined by flow cytometry. (C) Bacterial burden in the lung was determined by plating BAL fluid for bacterial CFUs. Data are representative of two (A–C) experiments. (Mean ±

s.d. in A. Median in B, C). n.d., not detectable. WT, B6.SJL. *, $p<0.05$. **, $p<0.01$. ***, $p<0.005$. (Statistical analysis: Mann-Whitney U test).

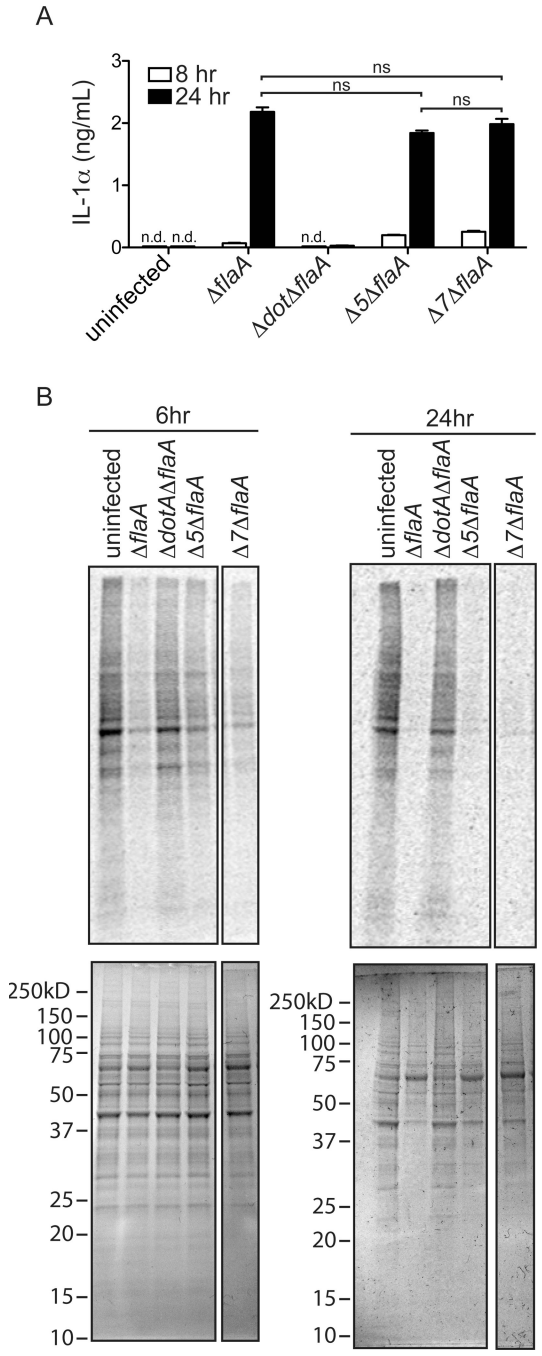

Figure 5.

L. pneumophila mutants lacking bacterial effectors known to block translation have no defect in IL-1α production. (A) Wild-type B6 bone marrow derived macrophages were infected with the indicated strains of *L. pneumophila* (LP02) at a MOI of 1. 8hrs and 24hrs post-infection cell supernatants were collected and IL-1α levels were determined by ELISA. (B) Wild-type bone marrow derived macrophages were infected with the indicated strains of *L. pneumophila* (MOI=3) and at 6hrs (left panels) and 24hrs (right panels) post-infection cells were incubated with ^{35}S-methionine for one hour followed by lysis in RIPA buffer. Gels were stained with coomassie blue to visualize equal loading (bottom panels) and global translation levels were determined by autoradiography (top panels). Intervening lanes on gel

were removed for simplicity. Data are representative of two (B) or three (A) experiments. (Mean ± s.d. in A). n.d., not detectable. ns, not significant. (Statistical analysis: Mann-Whitney U test).

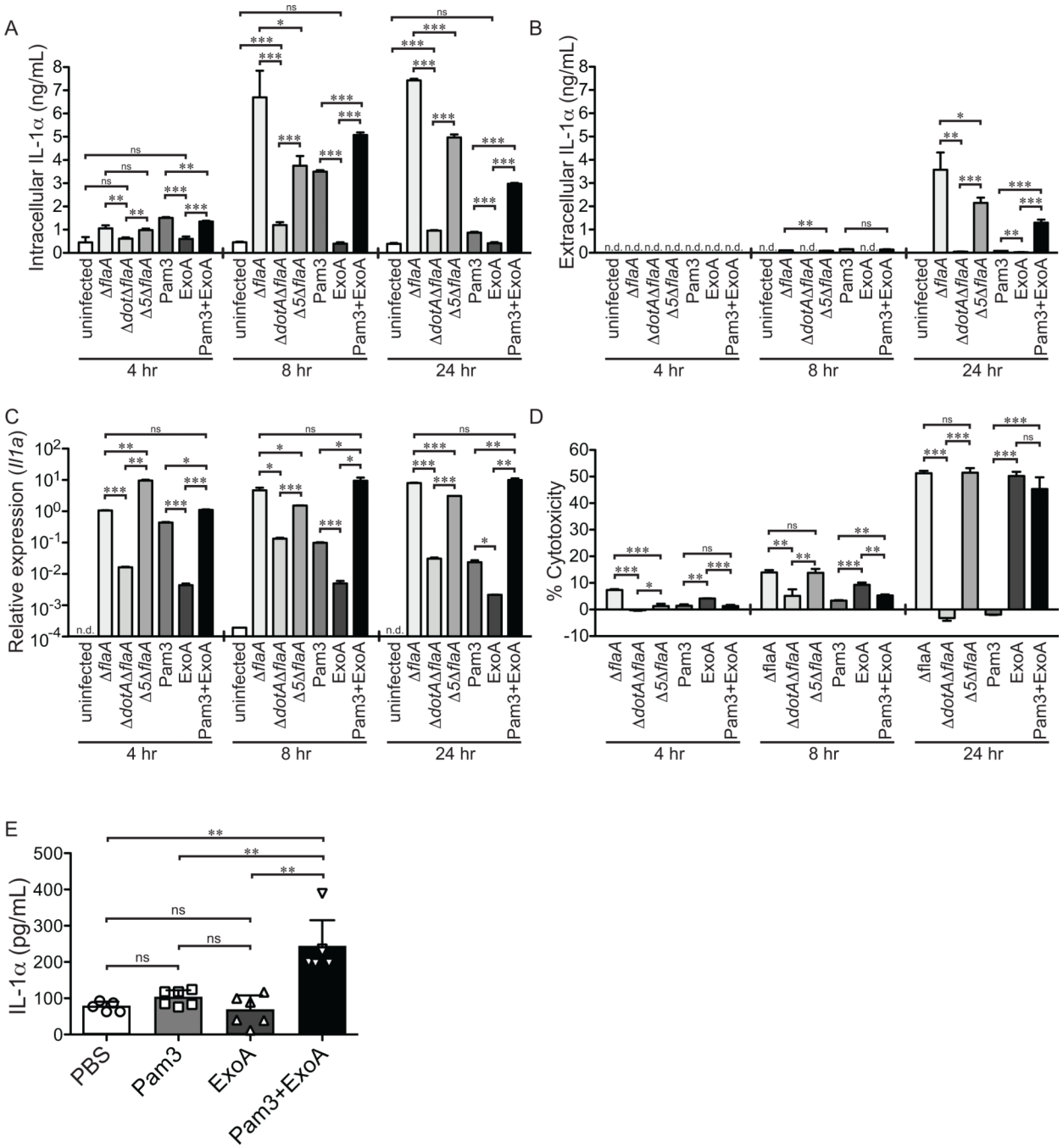

Figure 6.

Translation inhibition in conjunction with TLR activation is sufficient to induce the production of IL-1α both *in vitro* and *in vivo*. (A–D) Wild-type B6 bone marrow derived macrophages were infected with the indicated strains of *L. pneumophila* (LP02) or treated with Pam3CSK4 (10μg/mL), Exotoxin A (50ng/μL) or both Pam3CSK4 and ExoA. Samples were collected 4, 8, or 24 hours post-treatment. (A) Cells were lysed with RIPA buffer and intracellular IL-1α levels were determined by ELISA. (B) Extracellular IL-1α levels were determined by performing ELISA on cell supernatants. (C) *Il1a* transcript levels were assayed by quantitative reverse transcriptase PCR. (D) Cell cytotoxicity was determined by measuring the release of Lactate Dehydrogenase into cell supernatants and values were

normalized to an untreated control and a 100% lysis control where cells were treated with 1% TritonX-100 for 30 minutes. (E) Wild-type B6 mice were treated intranasally with Pam3CSK4 (10μg/mouse), Exotoxin A (2μg/mouse) or both in 20 μL of PBS. Bronchoalveolar lavage was performed 24hrs post-infection. IL-1α levels in BAL fluid were determined by ELISA. Data are representative of three (A–E) experiments (mean ± s.d. in A-E). Pam3, Pam3CSK4. ExoA, Exotoxin A. n.d., not detectable. ns, not significant.*, $p<0.05$. **,$p<0.01$. ***,$p<0.001$. (Statistical analysis: Unpaired T-Test (A–D), Mann-Whitney U Test (E)).